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TECHNICAL MANUSCRIPT 517

OBSERVATIONS ON SPIRACULAR BEHAVIOR
IN Aedes MOSQUITOES

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DEPARTMENT OF THE ARMY
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Plant Pathology Division
PLANT SCIENCES LABORATORIES

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ABSTRACT

An apparatus was constructed to allow simultaneous observation of the thoracic spiracles of mosquitoes and to permit recording of the behavior observed. A microenvironment of controlled composition and relative humidity was provided. In Aedes aegypti and triseratus at rest in air the thoracic spiracles constantly open and close synchronously, although the degree of opening is minimal. Stationary flight, carbon dioxide, or hypoxia cause much more extensive synchronized opening. It was suggested that the thoracic compressions and extensions of the head noted in flight or hypoxia are possible ventilatory mechanisms. These were unrelated to the frequency of spiracular opening and closing. Decapitation eliminated spontaneous locomotor activity and this resulted in more conservative spiracular behavior.

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I. INTRODUCTION*

The function of spiracles in respiration has been of considerable interest to those viewing insects' overall management of water reserves,^{1,2} insect temperatures in flight and at rest as they differ from the ambient,³ and in their rate-limiting effects on respiration itself.^{4,5} Another important consideration has been the coordination of spiracular opening and closing with ventilatory movements, especially in flight.⁶⁻⁹ Ideally, the spiracular valves would eliminate undesirable water loss while meeting respiratory needs. The spiracles must therefore effectively meet many exogenous, endogenous, and often conflicting physiological requirements if the insect is to survive its many environmental situations.

The numerous studies on spiracular behavior include the pioneering work of Hazelhoff¹⁰ and Wigglesworth¹¹ on cockroach and flea spiracles, which formed much of the basis of present knowledge of the role of carbon dioxide and oxygen in spiracular regulation. Schneiderman,¹² Schneiderman and co-workers,¹³ and Buck⁴ provided quantitative and theoretical information on tracheal gas dynamics and kinetics. Hoyle,^{14,15} Miller,^{7,16} and Van der Kloot¹⁷ used electrophysiological techniques in evaluating such aspects of respiration as ventilation, myogenic behavior of the spiracular closer muscle and the mechanism of carbon dioxide and oxygen action upon respiration.

Reports on respiration in imaginal mosquitoes deal almost exclusively with the measurement of oxygen uptake.^{18,21} However, some information is available in regard to the structure and function of adult mosquito spiracles.²⁰⁻²³ Clements²⁴ summarized the literature and discussed the probable relationship of spiracular regulation of respiration and water loss to survival and longevity.

It was therefore considered worthwhile to examine systematically the opening-closing cycle of mosquito spiracles (i.e., "spiracular behavior") in view of their fundamental importance in physiological adaptation to the environment.

Broadly stated, the objectives of this research were to (i) develop means of observing, measuring, and recording the behavior of mosquito spiracular valves, and (ii) gather observations relevant to spiracular behavior in atmospheres of varying composition and humidity and to certain treatments and nutritional states of the mosquito. This report is confined to describing the techniques employed in studying spiracular behavior and to presenting qualitative information on the functioning of the spiracular valves. Other aspects will be reported separately later.

* This report should not be used as a literature citation in material to be published in the open literature. Readers interested in referencing the information contained herein should contact the senior author to ascertain when and where it may appear in citable form.

II. MATERIALS AND METHODS

The apparatus employed in these studies consisted of three component systems necessary to (i) observe simultaneously two opposite spiracles, (ii) provide desired atmospheric and microenvironmental conditions, and (iii) record the spiracular behavior thus observed. The first requirement was satisfied by mounting two Leitz microscopes in opposed horizontal positions on an appropriate metal support. A modified mechanical stage for positioning the insect of study was attached to the same support and placed between the microscopes. Each microscope was equipped with reflected-light illumination (Leitz Ultropak).

A multi-axis stage was designed to position a mosquito between the microscopes (Fig. 1 and 2). This essentially consisted of a typical rack-and-pinion mechanical stage to which a U-shaped plastic superstructure was attached. The open end of the U supported a double ring that allowed it to rotate on its lateral axis. The ring consisted of an outer rim and an inner lining capable of rotating within the rim. The mosquito was held with a blunted 22-gauge hypodermic needle through which a vacuum was applied. The base of the needle fitted snugly into the inner lining of the double ring, and the point protruded to the center. Use of the mechanical stage allowed a mosquito to be moved vertically and horizontally in one plane; with the ring, it could be rotated and moved through the focal plane of the microscopes. Finally, the mosquito could be rotated laterally simply by turning the needle. Thus the multi-axis stage afforded close and easy examination of all external surfaces of the mosquito.

Experimental atmospheric conditions were provided as diagrammed in Figure 3. Appropriate gas mixtures were prepared from steel cylinders of dry oxygen, nitrogen, carbon dioxide, and air. Rates of flow were measured with flowmeters accurate to $\pm 5\%$ and adjusted with Hoke-type needle valves. Gas temperature was best controlled by thermostat to maintain the laboratory at the desired temperature. Any proportion of the gas mixture could be routed through a water tank to establish a desired relative humidity. Relative humidity was measured with $3/4$ - by $1\ 3/4$ -inch electronic humidity-sensing elements* at two points in the gas delivery system: the first was placed so as to measure the humidity in the gas delivery tube. The second was at the orifices of the gas delivery tube. Humidity readings were identical in both locations. The gas delivery tube was constructed with $1/4$ -inch O.D. glass tubing jointed and bent so as to bathe the test mosquito with two streams of gas directed from the antero-lateral aspects.

Because very small concentrations of carbon dioxide were needed, a separate delivery line for this gas was devised with a low-capacity flowmeter (10 to 250 cc/min); this was directly connected to the gas delivery tube.

* HygroDynamics Inc., Silver Spring, Md.

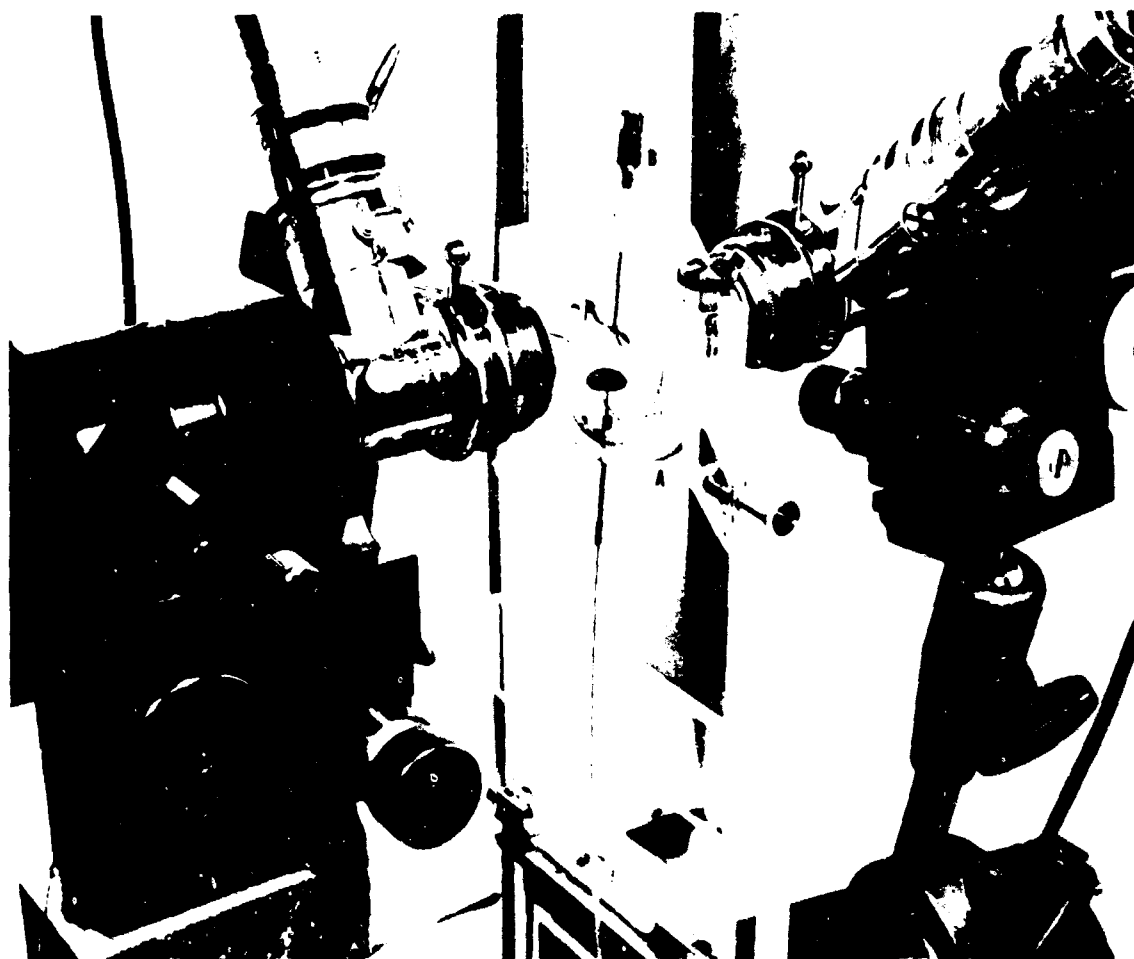


FIGURE 1. Horizontally Mounted Microscopes. (A) Mechanical stage. (B) Hypodermic needle and saddle. A vacuum is maintained through the needle.

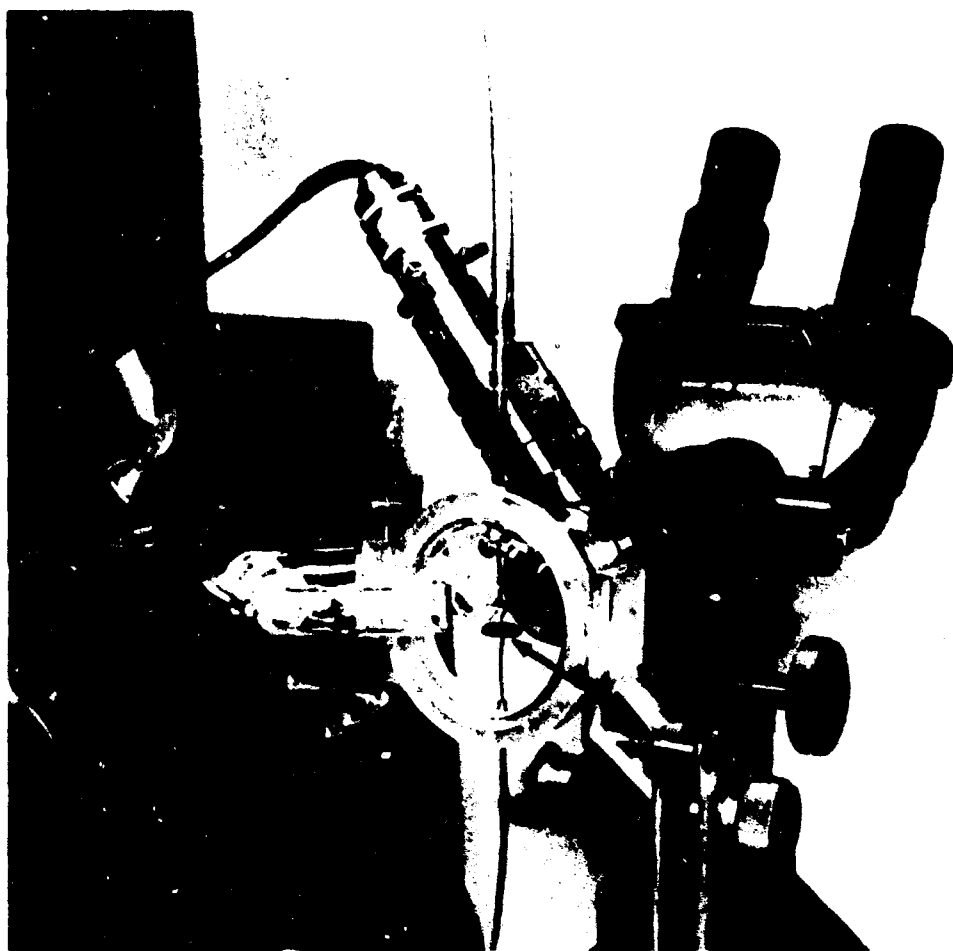


FIGURE 2. Binocular Microscope and Stage. Arrow points to movable pedestal. Note gas delivery tube.

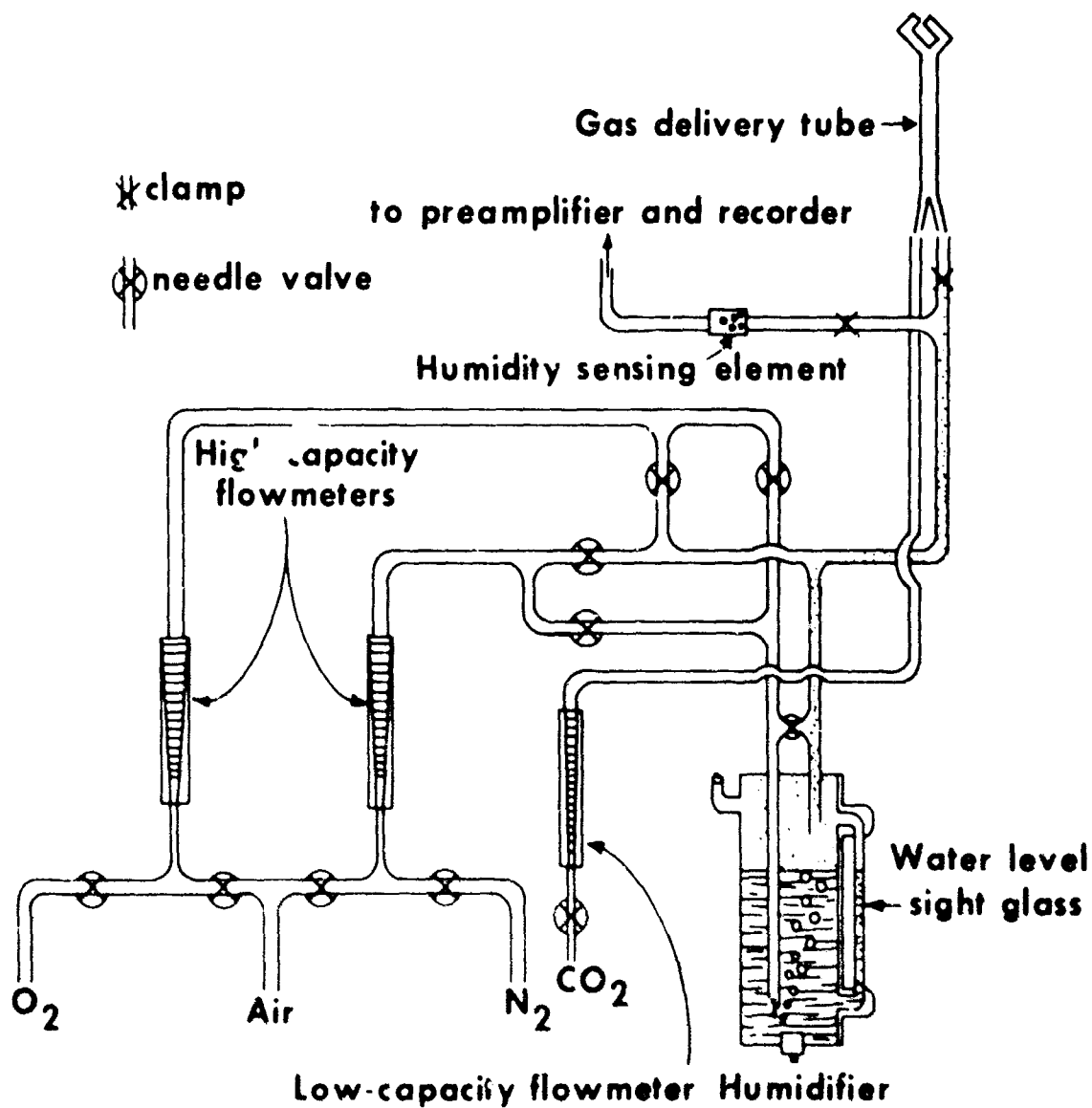


FIGURE 3. Diagram of the Gas-Mixing, Measuring, and Delivery Apparatus.

Oscillograms of spiracular behavior were obtained with a two-channel pen recorder* activated by push-button controls directed through an amplifier (Fig. 4). The push-button control mechanism allowed an observer three arbitrary positions for translating spiracular behavior into a permanent record for later analysis: a closed spiracle and two magnitudes of spiracular opening were thus recognized. When the spiracle closer muscle partially relaxed, the spiracular lips parted proximally in scissors-like fashion; this was the first position and was indicated by pushing and holding the first of three buttons for the time the spiracle was in that position. The second position was when the spiracular lips opened to the extent that they no longer touched at any point (i.e., were fully opened); in response the observer pressed and held the second button. The third button was reserved for recording the occurrence of brief spasms of spontaneous locomotor activity; it was depressed only momentarily, then observation and recording of spiracular behavior continued.

Oscillograms were scored by tabulating values of duration and amplitude of spiracular opening. Specifically, the following were noted: (i) frequency of spiracular opening (i.e., the number of times a spiracle opened from the closed position per minute); (ii) number of seconds per minute spiracles were partially open (first magnitude); (iii) number of seconds per minute spiracles were fully open (second magnitude); and (iv) frequency of spontaneous locomotor activity per minute. Four indexes were therefore used to describe spiracular behavior. These are abbreviated as follows: "Freq." refers to frequency of spiracular opening per minute; "toD" is seconds per minute spiracles were partially open; "toF" refers to seconds per minute spiracles were fully open; "toT" is the sum of "toD" and "toF" and thus describes total duration of opening regardless of amplitude. "Activity" refers to the frequency of spontaneous locomotor activity per minute.

The insects used in this and subsequent studies were reared in trays of 5,000 or 10,000 larvae each. Exclusively, those A. aegypti larvae that pupated on the 6th day of life (day 6 pupae) and day 7 pupae of A. triseriatus were allowed to emerge in pint ice-cream cartons of 50 individuals or gallon containers of 300 to 500 individuals each. Ages of adults are given ± 12 hours. Unless stated otherwise, each carton of adult mosquitoes had constant access to a cotton pad containing 30 ± 2 cc of 10% sucrose solution. Pads were changed daily. Mosquitoes were maintained at 80 F and $80 \pm 5\%$ relative humidity until individually used in observations on spiracular behavior.

The following protocol was employed in preparing each mosquito for observation. Mosquitoes were gently aspirated from an appropriate container into a $\frac{1}{2}$ - by 2-inch plastic vial and chilled at 25 ± 5 F for $1\frac{1}{2}$ to 2 minutes in the freezing compartment of a refrigerator. The immobilized insect was then removed and one or two prothoracic legs were amputated with micro-scissors to prevent occlusion of the spiracle or spiracles to be observed.

* Brush Instruments, Cleveland, Ohio.



FIGURE 4. Equipment for Recording Behavior of the Spiracular Valves.
(A) Push-button controls for two-channel pen oscillograph.
(B) Amplifier. (C) Two-channel pen oscillograph. (D) Oscillogram
made in observing behavior of opposite thoracic spiracles in Aedes
egypti.

A vacuum of 18 to 22 lb/in² was continuously applied to the insect's mesonotum through a hypodermic needle, thus firmly attaching the mosquito while allowing its head, abdomen and appendages freedom of movement. The mosquito was then placed on the mechanical stage and oriented to bring its spiracles into view.

Observations were begun within 2 to 3 minutes after the mosquito was mounted. This allowed it to become acclimated to the experimental conditions. Each mosquito was observed continuously for 3 minutes unless stated otherwise. The first minute represented spiracular behavior as it occurred in air, thus serving as a control observation; the second 2 minutes represented spiracular behavior in atmospheres of various compositions.

The total rate of gas flow in all experiments was standardized at 5,000 cc per minute and temperatures were maintained at 70 to 73 F.

Early bench trials indicated that observation of abdominal spiracles would not be possible at a magnification of less than 200 diameters. This required objectives of short focal length. Even normal abdominal movements would place spiracles out of focus. Attention was therefore directed exclusively upon the two pairs of thoracic spiracles. With 6.5X objectives and 15X field eyepieces, an optimal free working distance of 16.5 mm was achieved with a comfortable overall magnification of 97.5 diameters. Spiracle length and width in the open and closed position were measured with an ocular micrometer at a magnification of 165 diameters.

III. RESULTS

At this point it would be well to review briefly Hassan's²² findings on spiracle structure among the Culicidae. The thoracic spiracles of Anopheles and Culiseta were described as functionally consisting of a flexible caliper-like margin to which the peritremes, or "lids," are fixed. The closer muscle lies at the base of the caliper. Spiracle opening results from relaxation of the closer muscle and the elasticity of the caliper. The basic mechanics of this system are similar to that of saturniid pupae¹² and in fact to that of many other insects.^{8,9}

It is now desirable to describe the behavior of thoracic spiracles in resting mosquitoes under standard conditions in air. As in the largest spiracles of Xenopsylla,¹¹ thoracic spiracles of A. triseriatus and A. aegypti are always functional, because they continuously and rhythmically open and close, which gives the appearance of twitches. The frequency of such opening may vary among individuals from approximately one to more than eight openings and closings per minute. The higher frequencies of opening were generally observed in newly emerged adults. Amplitude of spiracular opening also varies greatly among individual mosquitoes. In air, the spiracular lips separate proximally to the closer muscle, usually about 1/8 to 1/4 of the length of the spiracle. In A. triseriatus, thoracic spiracle length varies from 0.13 mm to 0.17 mm. Thus, an elliptical orifice of approximately 0.016 to 0.042 mm long and 0.008 to 0.021 mm wide is produced with each opening. In some individuals, visible separation of spiracular lips was not observed, even though rhythmical spiracular movement on the dorso-ventral axis was apparent. Despite the lack of visible evidence, it is doubtful that there was no opening. This is considered spiracular opening of the first magnitude, or toD.

A more extensive spiracular opening than that described above will occur during a period of spontaneous locomotor activity, in the presence of small concentrations of carbon dioxide, or with hypoxic conditions. In these instances opening may continue along the entire length of the spiracle. The width of the opening is again highly variable, from 0.02 to 0.063 mm. Maximum spiracular opening averages about 0.05 mm in width. Complete longitudinal spiracular opening, regardless of the width of that opening, was recorded and tabulated as an opening of the second magnitude, or toF. This categorization of spiracular opening may have physiological significance if "pore diffusion" operates in mosquito thoracic spiracles. In "pore diffusion" the rate of gas diffusion is proportional to the perimeter rather than the area of a spiracle.^{4,5}

Before attempting experimental work involving various atmospheres and relative humidities, it was necessary to determine whether gas flow over a mosquito at 5,000 cc/min influenced normal spiracular behavior. Accordingly, behavior of the right mesothoracic spiracles was recorded in A. triseriatus subjected to 5,000 cc/min air flow or to no air flow at all. Care was taken to insure that relative humidity of the air

stream matched that of the room. Results of one such experiment with 4-day-old adult A. triseriatus are shown in Table 1; these provided evidence that no significant difference between treatments existed ($P > 0.05$). It thus seemed unlikely that the experimental procedures described above would produce spiracular behavior that differed from normal.

TABLE 1. SPIRACULAR BEHAVIOR OF 4-DAY-OLD Aedes triseriatus IN STILL AIR AND IN AN AIR FLOW OF 5,000 cc/MIN AT 70 F^a

Treatment	No. of Insects	Freq.	toF	toT	Activity
5,000 cc/min	12	4.5±0.69	4.5	42.7±3.0	0.55
Still air	12	3.8±0.87	6.2	42.7±3.1	0.66

$P > 0.05$

a. Values represent means of 3-min observations ± standard errors.

For guidance in future work, it was of interest to know if the opening and closing of thoracic spiracles were synchronized. Two approaches in examining spiracular synchronization were used. In the first, two adjacent spiracles were simultaneously observed by orienting the mosquito in the microscope field and recording the action of one spiracle while mentally noting the behavior of the other. This visual correlation method was frequently used throughout the course of the present study under many experimental conditions. These observations indicated that adjacent mesothoracic and metathoracic spiracles performed in strict synchrony with regard to frequency and amplitude in atmospheres of air, air plus carbon dioxide, low oxygen, and stationary flight. The second approach examined spiracular behavior in two opposite thoracic spiracles and required two observers. Table 2 includes data so obtained in experimental conditions of low oxygen, air plus 4% carbon dioxide, air alone, and in the use of decapitated mosquitoes. Visual inspection of the resulting oscillograms showed that opposite spiracles indeed acted in concert under all conditions employed. The values representing duration of spiracular opening differ only slightly. In particular, these were largely confined to differences in amplitude of opening. When the total duration of both amplitudes of spiracular opening was considered (toT), the differences between spiracles became negligible, and were therefore thought to be the result of observational error. Thus, the short muscle relaxations resulting in twitches, and the more extensive relaxations giving full spiracular opening, occurred simultaneously in all thoracic spiracles and suggest that a central pacemaker exists.

TABLE 2. SYNCHRONIZATION OF OPPOSING THORACIC SPIRACLES
IN A. triseriatus AND A. aegypti

Treatment	Species	Spiracles Observed ^a /	Freq.	toF	toT
12% oxygen N ^b = 8	<u>A. triseriatus</u>	RMSS+RMTS	6.68	16.00	35.25
		LMSS+LMTS	6.68	12.87	36.00
10% oxygen N = 9	<u>A. aegypti</u>	RMSS+RMTS	9.44	11.82	49.70
		LMSS+RMTS	9.99	12.88	49.21
10% oxygen N = 9	<u>A. aegypti</u>	RMSS+RMTS	9.71	9.72	28.88
	Decapitated	LMSS+LMTS	9.60	7.16	28.15
Air alone N = 10	<u>A. aegypti</u>	RMSS+RMTS	6.20	6.30	37.60
		LMSS+LMTS	6.20	6.30	37.60
1% CO ₂ in air N = 10	<u>A. triseriatus</u>	RMSS+RMTS	4.25	13.30	34.75
		LMSS+LMTS	4.25	16.00	35.00

- a. RMSS = right mesothoracic spiracle.
RMTS = right metathoracic spiracle.
LMSS = left mesothoracic spiracle.
LMTS = left metathoracic spiracle.

- b. Number of experiments.

Observations were made to determine if ventilatory movements took place in imaginal mosquitoes at rest and in stationary flight. To induce stationary flight, the stage pedestal (Fig. 2) was withdrawn. Systematic data were not gathered on ventilation in atmospheres containing carbon dioxide or low oxygen, but rather were observed as the experiment progressed. Possible ventilatory movements were noted among the following and are listed according to their frequency of occurrence: (i) telescopic abdominal compression and peristalsis; (ii) thoracic compression, very weak in A. triseriatus and more evident in A. aegypti; (iii) rhythmic extensions and contractions of the head and neck. No ventilatory movements were noted in resting insects in air, but all three were occasionally apparent in atmospheres of low oxygen. Carbon dioxide (1%) had no apparent effect on ventilatory movements. Mosquitoes often showed abdominal contractions and/or pulsations following several minutes of stationary flight and this often continued up to 30 seconds beyond cessation. No correlation between the postulated ventilatory movements and spiracular behavior was observed. Spiracular opening and closing in flying A. triseriatus was more variable than that in A. aegypti. In the latter, thoracic spiracles opened maximally in flight and held that position until cessation of flight, with few exceptions. In fact, flight and other spontaneous locomotor activity was usually anticipated in A. aegypti by full spiracular opening. However, in A.

triseriatus the spiracular movements were more often a reflection of pre-flight rhythm, with greater amplitude of opening. In a stationary flight of 12 to 15 minutes' duration, amplitude of opening would gradually increase to maximum at about 5 minutes and thereafter progressively decrease. In some individuals, sustained spiracular opening was noted for the first 10 minutes of a 15-minute flight.

In A. aegypti there was a strong correlation between spontaneous locomotor activity and extended spiracular opening. It was because of this activity that A. aegypti showed less conservative spiracular behavior and proved less easy to work with than A. triseriatus. Galun²⁰ obtained extremely variable oxygen consumption values with A. aegypti. She found that decapitation reduced variation and activity but that, surprisingly, oxygen uptake was increased. Efforts were therefore made to measure the influence of spontaneous locomotor activity on spiracular behavior in both A. aegypti and A. triseriatus. To eliminate spontaneous activity, mosquitoes were decapitated with microscissors immediately prior to mounting them before the microscopes. Twenty to 30% of the operations were incomplete in that small portions of the head remained. Decapitated insects were compared with controls exposed to similar experimental conditions (Table 3).

TABLE 3. EFFECTS OF DECAPITATION ON AMPLITUDE AND DURATION OF SPIRACULAR OPENING AND ON SPONTANEOUS LOCOMOTOR ACTIVITY IN A. AEGYPTI AND A. TRISERIATUS

Species and Treatment		Freq.	toF	toT	Act	N ^{a/}
<u>A. aegypti</u> in $\frac{1}{2}$ % CO ₂	Intact	1.2	56.1	57.9	1.3	10
	Decapitated	4.7	32.4	47.2	0.0	10
<u>A. triseriatus</u> in $\frac{1}{2}$ % CO ₂	Intact	2.0	29.9	39.1	0.7	39
	Decapitated	4.0	23.5	37.0	0.0	21
<u>A. aegypti</u> ^{b/} in 10% O ₂	Intact	9.7	11.8	49.7	1.5	10
	Decapitated	9.7	9.7	28.9	0.0	10

a. Number of experiments.

b. Newly emerged, 5 to 15 hours old.

Elimination of spontaneous locomotor activity and more conservative spiracular opening was the immediate result of decapitation in both species. A. aegypti controls showed higher frequencies of activity than A. triseriatus; following decapitation, spiracular response to carbon dioxide or hypoxia was lowered in the former species. Thus the greater degree of spiracular opening normally exhibited by A. aegypti was probably the result of its greater reaction to environmental stimuli and possibly of a greater inherent metabolism.

IV. DISCUSSION

Because of the infinite number of spiracular positions between complete closure and full opening, the present three recognized classes may seem rather arbitrary. However, such categorization was clearly the most practical solution to measuring the quality of spiracular opening. This was because several observers could rather consistently agree upon which class of opening a spiracle assumed at a given moment, and because spiracular behavior fell quite naturally into such categories under normal atmospheric conditions. Only in hypoxic or high carbon dioxide atmospheres did the spiracles show intermediate grades of opening for significant periods of time. It bears notice that the thoracic spiracles of mosquitoes are very large compared with those of the abdomen; associated with this fact is the observation that they operate at two quite different levels of activity: the very narrow openings of short duration seen in the resting insect in air, and the much more extensive opening seen in stationary flight. Clearly, the thoracic spiracles are an adaptation to meet the needs of respiration in flight.

Although extensive studies were not performed on ventilation, our observations confirm that abdominal compression plays no part in the respiration of flying mosquitoes. Galun and Fraenkel²¹ noted abdominal peristalsis in A. aegypti but concluded that this was in fact a manifestation of the heartbeat. Christophers²⁵ pointed out that tracheal communication between the thorax and abdomen was negligible. However, the thoracic compression and the extensions and retractions of the head noted in this study may ultimately be shown to provide tidal ventilation, or at least to aid mass transfer of respiratory gases. It should be emphasized that such movements do not occur in mosquitoes at rest in air. In the honeybee, tidal ventilation occurs in the insect at rest; directed airflow may occur with activity.⁴ Neville²⁶ called attention to the fact that the distance that myogenic fibrillar flight muscles contract is less than in non-fibrillating muscle; consequently, thoracic compression and expansion is much reduced in the Diptera and Hymenoptera. However, thoracic compression and extensions of the head and neck play an important part in the respiration of certain Odonata and Orthoptera.^{7,16} There are a plethora of

ventilating mechanisms, which vary within major groups (e.g., Odonata) as well as between them; these are summarized by Buck⁴ and Miller.⁵ Ventilation in mosquitoes deserves further study.

Our suggestion that there is central control of spiracular opening and closing rests entirely upon our observation of the strict synchrony of thoracic spiracles. It is difficult indeed to visualize each of four spiracles acting in such unison from the dictates of solely extrinsic stimuli. Synchronization of the spiracles, however, is not universal among the Insecta. In saturniid pupae, Schneiderman¹² observed non-synchronous spiracular behavior. Van der Kloot¹⁷ demonstrated that motor impulses to the spiracular closer muscles of these forms were indeed asynchronous. On the other hand, in those forms showing ventilation in flapping flight, many kinds of precise and complicated synchronizing mechanisms coordinate the inspiratory spiracles, expiratory spiracles, and ventilation. A respiratory pacemaker was recently demonstrated in the cockroach.²⁷

That decapitated mosquitoes showed more conservative spiracular behavior than intact controls does not agree with Galun's²⁰ finding that such mosquitoes had a higher respiratory rate. Galun stated that upon decapitation, the average spiracle aperture increased, and complete spiracle closure did not occur. Spontaneous locomotor activity was eliminated and rhythm of opening and closing remained similar to that in intact controls. Galun's observations were made in air; ours were made under hypoxic atmospheres or those containing carbon dioxide, which may account for the disparity. Both carbon dioxide and hypoxia have well-known sensory effects upon the spiracle closer muscle itself (peripheral effects) and on the central nervous system; these atmospheres may well have stimulated partially relaxed spiracular closer muscles once more into normal functioning.

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<p>An apparatus was constructed to allow simultaneous observation of the thoracic spiracles of mosquitoes and to permit recording of the behavior observed. A micro-environment of controlled composition and relative humidity was provided. In <u>Aedes aegypti</u> and <u>triseriatus</u> at rest in air the thoracic spiracles constantly open and close synchronously, although the degree of opening is minimal. Stationary flight, carbon dioxide, or hypoxia cause much more extensive synchronized opening. It was suggested that the thoracic compressions and extensions of the head noted in flight or hypoxia are possible ventilatory mechanisms. These were unrelated to the frequency of spiracular opening and closing. Decapitation eliminated spontaneous locomotor activity and this resulted in more conservative spiracular behavior.</p>		
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